PHOSPHOROLYSIS OF OLIGORIBONUCLEOTIDES BY POLYNUCLEOTIDE PHOSPHORYLASE

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The enzyme polynucleotide phosphorylase catalyzes the over-all reaction described in Equation 1.

$$n \text{ nucleoside-PP} \xrightleftharpoons{Mg^{++}} (\text{nucleoside-P})_n + n \text{ inorganic P} \qquad (1)$$

This reversible reaction has been demonstrated with enzyme preparations from *Azotobacter vinelandii* (1, 2) and *Escherichia coli* (3). In the forward direction the enzyme catalyzes the formation of polyribonucleotides similar in structural details to natural RNA[1] (5–8). In the reverse reaction polyribonucleotides are phosphorolyzed to yield nucleoside diphosphates. The phosphorolysis of synthetic polymers made by the enzyme and the phosphorolysis of ribonucleic acids isolated from various natural sources have been studied by Ochoa and coworkers with the *A. vinelandii* enzyme (2, 9), by Littauer and Kornberg with a preparation from *E. coli* (3), and by Heppel.[2] Highly polymerized RNA preparations were phosphorolyzed at slower rates than the synthetic polymers; however, commercial RNA and RNA "core," the limit polynucleotides obtained after exhaustive

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[1] The following abbreviations are used: adenosine and uridine units are represented by A and U, respectively; 5′-diphosphates of adenosine and uridine, ADP and UDP; 5′-nucleoside diphosphate, NDP; 5′-monophosphates of adenosine and uridine, AMP and UMP; 5′-triphosphate of adenosine, ATP; ribonucleic acid, RNA; polyadenylic acid, poly A; polyuridylic acid, poly U; mixed polymer of adenylic and uridylic acids, poly AU; tris(hydroxymethyl)aminomethane, Tris; reduced diphosphopyridine nucleotide, DPNH; inorganic orthophosphate, P_i. Small polynucleotides are designated by a system proposed by Markham and Smith (4). A phosphate group is designated by "p"; when placed to the right of a nucleoside symbol, the phosphate is esterified at C-3′ of the ribose moiety; when placed to the left of the nucleoside symbol, the phosphate is esterified at C-5′ of the ribose moiety. Thus, pApA is a dinucleotide with 1 phosphate monoesterified at C-5′ of an adenosine residue and a phospho diester bond between C-3′ of that same adenosine residue and C-5′ of the other adenosine group. The symbol -cyclic-p is used to designate a terminal 2′,3′-cyclic phospho diester moiety on a polynucleotide. Thus, UpU-cyclic-p is a dinucleotide with a 2′,3′-phosphate at the terminal ribose.

[2] Cited by Littauer and Kornberg (3) and Ochoa (9).

digestion of RNA with pancreatic ribonuclease, were attacked very slowly, if at all. In the light of these data it was not possible to define any requirements, by polynucleotide phosphorylase, for specific structural details in the polynucleotide substrates. The preparations used were not of uniform, known chain length and, in addition, the end group structures of the natural RNA preparations have not been clearly established.

Heppel and coworkers recently described methods for the preparation of several homologous series of well characterized oligoribonucleotides (7, 8, 10). This made it possible to investigate the specificity of the phosphorolysis reaction. The experiments to be described in this paper indicate that polynucleotides with C-5′ phospho monoester end groups are readily phosphorolyzed although those bearing C-3′ phospho monoester end groups are resistant to enzymic attack. The phospho monoester at C-5′ is not essential for activity, however, since oligonucleotides with no monoesterified phosphate groups, such as trinucleoside diphosphates, are phosphorolyzed. In addition, it was found that dinucleotides and dinucleoside monophosphates are not attacked by polynucleotide phosphorylase, and these compounds accumulate as resistant end products when the phosphorolysis of the larger oligonucleotides is studied.

A preliminary report of these findings has been made (11).

EXPERIMENTAL

Materials—Polynucleotide phosphorylase was purified from *E. coli* by Dr. R. J. Hilmoe according to the procedure of Littauer and Kornberg (3). The fraction used is described as Ethanol I (3) and was dialyzed before use. The preparation contained 1 mg. of protein per ml. and had a specific activity of 15, determined by the "exchange" assay (Assay C) (3). The preparation of *A. vinelandii* polynucleotide phosphorylase was kindly supplied by Dr. S. Ochoa. The fraction was an eluate from $Ca_3(PO_4)_2$ gel (12), contained 7.4 mg. of protein per ml., and had a specific activity of 40 as measured with the "exchange" assay (Assay 2) (2). Crystalline bovine pancreatic ribonuclease was a commercial preparation (Armour, lot No. 1044). Phosphomonoesterase was fractionated from human seminal plasma (4) after a preliminary treatment with protamine to remove nucleic acid material. The specific activity of this preparation was 3.7×10^3 units per mg. of protein (1 unit of enzyme liberates 1 μmole of inorganic phosphate per hour per 1.2 ml. of a reaction mixture containing 26 μmoles of AMP, in acetate buffer, pH 5.2). This enzyme preparation dephosphorylated dinucleotides more slowly than mononucleotides. Therefore, for the preparation of dinucleoside monophosphates a great excess of enzyme was used. Even more enzyme was required to dephosphorylate oligonucleotides of chain length greater than 2. The preparation used in these experi-

ments was relatively free from phosphodiesterase activity, but, when it was used in the high concentrations required to dephosphorylate oligonucleotides, appreciable diesterase activity was noted. The nuclease from guinea pig liver nuclei, which liberates small polynucleotides with C-5′ phosphate end groups from poly A, has been described by Heppel and coworkers (10).[3] A commercial preparation of lactic dehydrogenase (Worthington, crystalline) which was contaminated with pyruvate kinase was used as a source of both of these enzymes. Myokinase (63 units per ml., 0.8 mg. of protein per ml.) was supplied by Dr. B. L. Horecker and was prepared according to the procedure of Colowick (13).

Polymers were prepared from nucleoside diphosphates with the polynucleotide phosphorylase of *E. coli* or *A. vinelandii* according to the procedure of Grunberg-Manago and coworkers (2), but the method of isolation was slightly modified. After polymer formation at pH 8, the reaction mixtures were adjusted to pH 7 with acetic acid. Polymer was precipitated with 3 volumes of ethanol, separated by centrifugation, dissolved in water, and reprecipitated in the same manner. The twice precipitated polymer was dissolved in water and the solution was deproteinized by shaking with 0.25 volume of chloroform and 0.1 volume of isoamyl alcohol (14). The aqueous solution of polymer was then dialyzed against cold, running, distilled water for 3 days and lyophilized. A sample of poly A prepared with the polynucleotide phosphorylase of *Micrococcus lysodeikticus* (15) was generously donated by Dr. R. F. Beers.

All the mononucleotides used were commercial preparations (Sigma). Phosphoenolpyruvic acid was prepared by Mr. William E. Pricer, Jr., and DPNH was donated by Dr. B. L. Horecker.

Paper Chromatography and Paper Electrophoresis—Several systems were used for the separation of mononucleotides and oligonucleotides on paper. Descending chromatography was carried out with the following solvent systems: System 1, isopropanol-water (70:30, v/v) with NH_3 in the vapor phase (16); System 2, isobutyric acid-1 M NH_4OH-0.2 M ethylenediaminetetraacetate (100:60:0.8, v/v/v) (17). For Solvent 1, Whatman No. 3MM paper was used and for Solvent 2, Whatman No. 3MM or Whatman No. 1. Electrophoretic separations (referred to as System 3) were carried out according to Markham and Smith (16) on strips (57 × 10 cm.) of Whatman No. 3MM paper saturated with 0.05 M ammonium formate buffer, pH 3.5. A potential of 1000 volts was applied across the paper. Purine- and pyrimidine-containing compounds were located on the paper strips with an ultraviolet light which was also used to photograph the strips. When these methods of separation were used in a preparative way or to obtain materials for enzymic treatment, the purine and pyrimidine derivatives

[3] We thank Dr. R. J. Hilmoe and Dr. L. A. Heppel for this preparation.

were eluted with water. When quantitative elution of the material was desired, the ultraviolet-containing areas were eluted with 0.01 N HCl for 6 hours at room temperature, and the concentration of the compound was determined by measuring the absorption of the eluate at an appropriate wave length. From the adjacent region of the paper strip, an area of identical size was cut out and eluted in order to correct for the ultraviolet-absorbing material present in the paper itself.

Assay Procedures—In the procedure used (3), the phosphorolysis of polynucleotides was carried out in the presence of inorganic P^{32}. The resultant labeled nucleoside diphosphate (see Equation 1) was separated from inorganic P^{32} by adsorption onto charcoal and its radioactivity was measured. The reaction mixtures (0.125 ml.) contained the oligonucleotide or polymer, enzyme, 5 μmoles of Tris buffer, pH 8.0, 0.5 μmole of $MgCl_2$, and 3.2 μmoles of P^{32}-labeled sodium potassium phosphate buffer, pH 7.4. After incubation at 37°, the reaction was stopped by adding 0.1 ml. aliquots to 1.0 ml. of cold, 2.5 per cent perchloric acid. Acid-washed Norit A (0.1 ml. of a 10 per cent suspension, w/w) was added to adsorb the nucleotides. After 10 minutes in the cold, the suspension was centrifuged and the charcoal was washed three times with 2.5 ml. portions of water. The charcoal was then suspended in 0.8 ml. of 50 per cent ethanol containing 0.3 ml. of concentrated NH_4OH per 100 ml. An aliquot of this suspension (usually 0.1 ml.) was placed on a copper planchet, dried, and the radioactivity determined with a thin window, gas flow counter. The total counts per minute incorporated into charcoal-adsorbable material were determined and from the specific radioactivity of the inorganic P^{32} the micromoles of phosphate incorporated were calculated. A self-absorption factor of 1.15 (3) was applied. Two control incubations, one containing no substrate and one containing no enzyme, were generally carried through the whole procedure with each experiment, and the results presented have been corrected for the small amount of radioactivity adsorbed onto the charcoal from these samples.

When the products of phosphorolysis were to be isolated, the reactions were carried out in the same manner but on a larger scale. Nucleotides were eluted from charcoal by four treatments with 0.8 ml. of ethanolic ammonia; the ethanol supernatant fluids were pooled and concentrated before chromatography.

The phosphorolysis of polynucleotides was also measured by determining the nucleoside diphosphate formed with the spectrophotometric procedure of Kornberg and Pricer (18). The application of this method to the determination of polynucleotide phosphorylase activity has been described by Ochoa and Heppel (6). Myokinase was added to the assay system to reconvert to ADP any AMP and ATP that had been formed in the original

incubation as a result of myokinase activity in the polynucleotide phosphorylase preparation. UDP was also measured by this method (19).

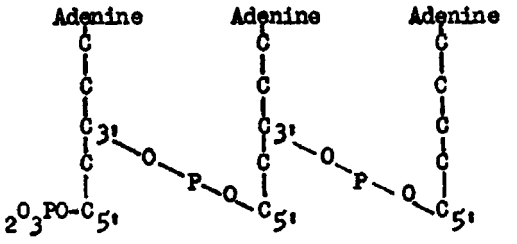
All optical measurements were carried out with a Beckman model DU spectrophotometer.

Preparation of Oligonucleotides—Five series of homologous oligonucleotides were prepared in order to study the specificity of polynucleotide phosphorylase. Each series included compounds of 2, 3, and 4 nucleoside residues and the structure of a representative member of each group is illustrated in Fig. 1. In every compound studied, the internucleoside links were 3′,5′-phospho diester bonds. The oligonucleotides used in this work were recently investigated by Heppel and coworkers (7, 8, 10), who isolated them from biosynthetic polymers and characterized them by methods developed by Markham and Smith (4), Volkin and Cohn (20), and others.

The compounds in Group I contained adenosine as the only nucleoside moiety and each of the three oligonucleotides had a monoesterified phosphate at C-5′ of the terminal nucleoside (7, 10). The structure of the trinucleotide, pApApA, is shown in Fig. 1. These compounds were described and characterized by Heppel and coworkers (7, 10) and are formed by the action of a nuclease from guinea pig liver nuclei on synthetic poly A. A typical preparation was carried out in the following way: The incubation mixture (30 ml.) contained 400 μmoles of $MgCl_2$, 800 μmoles of phosphate buffer, pH 7.2, 80 mg. of poly A (*M. lysodeikticus*), and 5 ml. of enzyme containing about 30 mg. of protein. After 6 hours of incubation at 37°, toluene was added to inhibit bacterial growth. After 24 hours of incubation the mixture was cooled, deproteinized with chloroform (14), and concentrated by lyophilization. The resulting solution was divided into eight equal portions and each was applied as a thin band to filter paper and chromatographed for 64 hours in System 1. The di-, tri-, and tetranucleotides (pApA, pApApA, and pApApApA) were eluted from the papers and the eluates were concentrated at 40° in a stream of air. The concentration of oligonucleotide was estimated by measuring the ultraviolet absorption of the eluate at 257 mμ and applying the extinction coefficient for adenylic acid.[4] The dinucleotide (pApA) that was obtained in this way was contaminated with ADP and inorganic P and was further purified by paper electrophoresis.

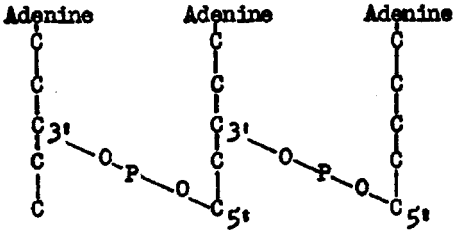
[4] It was assumed for this work that the molar extinction coefficient of an oligonucleotide is approximated by the sum of the molar extinction coefficients of its constituent nucleotides. For example, the concentration C of ApApUp in micromoles of oligonucleotide per ml., when the absorption is measured at pH 2, is given by $C = \text{Abs.}_{260}/(2(15.1) + 10.0)$, where the molar extinction coefficients for AMP and UMP are 15,100 and 10,000, respectively. Recent unpublished experiments by the present author indicate that, after alkaline hydrolysis, the absorption of pApApA at 257 mμ and pH 7 increases by approximately 15 per cent. Enzymic hydrolysis of dinucleotides in the deoxyribose series also results in an increased absorption (21).

The compounds of Group II were derived from those of Group I by the removal of the monoesterified phosphate at C-5′ by human seminal plasma phosphomonoesterase (7, 10). The structure of the trinucleoside diphos-



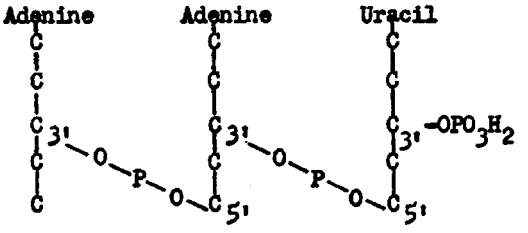
pApApA

Trinucleotide of Group I



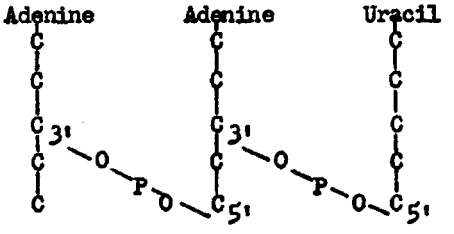
ApApA

Trinucleoside diphosphate of Group II



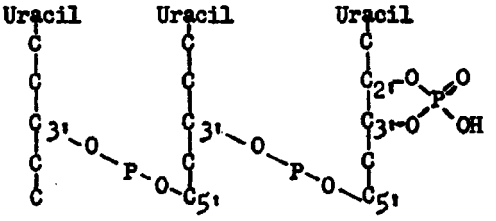
ApApUp

Trinucleotide of Group III



ApApU

Trinucleoside diphosphate of Group IV



UpUpU-cyclic-p

Trinucleotide of Group V

FIG. 1. Outline structures of the trinucleotides and trinucleoside diphosphates

phate (ApApA), which was derived from pApApA, is shown in Fig. 1. The di-, tri-, or tetranucleotide of Group I was incubated with monoesterase under the conditions described by Heppel and coworkers (7), and the cor-

responding nucleoside derivatives were separated by chromatography in System 1 (10). The products were obtained from the paper strips in the manner described for the compounds of Group I.

The three oligonucleotides in Group III were obtained by exhaustive digestion of poly AU with ribonuclease (8). The structure of the trinucleotide, ApApUp, is shown in Fig. 1. These oligonucleotides contained varying numbers of adenosine residues but each had a terminal uridine unit with a monoesterified phosphate at its C-3′. Thus the homologous di- and tetranucleotides were ApUp and ApApApUp, respectively. The electrophoretic and chromatographic properties of these compounds and the methods of identification have been recorded by Markham and Smith (4) and Volkin and Cohn (20). In a typical preparation, 15 mg. of poly AU were incubated with 0.6 mg. of ribonuclease for 16 hours at 37° in 0.05 M Tris buffer, pH 8 (total volume equal to 1.7 ml.). The mixture was deproteinized as described above (14), and the aqueous layer was concentrated, applied to paper, and chromatographed in System 1. The bands obtained were eluted and the absorption of the eluates at 260 mμ was determined. The sum of the extinction coefficients for each mononucleotide residue was used to estimate concentration of the oligonucleotide.[4]

The dinucleoside monophosphate ApU, trinucleoside diphosphate ApApU, and tetranucleoside triphosphate ApApApU of Group IV were obtained from the corresponding oligonucleotides of Group III by treatment with seminal phosphomonoesterase, exactly as described for the preparation of Group II from Group I. The properties of the compounds in Group IV are described in the literature (8, 4, 20) and differ from those of Group II in that the terminal nucleoside is uridine (see Fig. 1). The concentrations of these compounds were estimated as described for Group III.

The last homologous series of oligonucleotides (Group V) was obtained from the controlled digestion of poly U with small amounts of ribonuclease (7). Each member of the group contained exclusively uridine residues with a cyclic-terminal phospho diester moiety. The structure of the cyclic terminal trinucleotide, UpUpU-cyclic-p, is shown in Fig. 1. These compounds were separated by chromatography in Solvent 1 and were eluted from the papers with water. By applying the extinction coefficient for uridylic acid,[4] the concentrations of the oligonucleotides were determined by measuring the absorption of the eluates at 262 mμ.

Results

The trinucleotide and tetranucleotide of Group I, which had a phospho monoester group at C-5′, were readily phosphorolyzed by the polynucleotide phosphorylases of *E. coli* and *A. vinelandii* (Table I). The compounds

of Group III, which contained a phospho monoester moiety at C-3′, were, however, resistant to phosphorolysis by either of the enzymes (Table II). The data in Table II suggested that ApApApUp was very slowly cleaved by the *A. vinelandii* enzyme, but this was not confirmed by other studies with both enzyme fractions. Incubation was carried out as in Table II but for a period of 24 hours; chromatographic investigation of the reaction

TABLE I

Phosphorolysis of Compounds in Group I and II

Group No.	Substrate	*E. coli* experiment			*A. vinelandii* experiment		
		Substrate concentration*	Total radioactivity†	Rate of phosphorolysis‡	Substrate concentration*	Total radioactivity†	Rate of phosphorolysis‡
		μmole per ml.	*c.p.m.*		*μmole per ml.*	*c.p.m.*	
I	pApA	0.3	0	0.0	0.6	130	0.0
	pApApA	0.3	3470	0.6	0.6	3,700	0.7
	pApApApA	0.4	8910	1.4	0.7	12,780	2.6
II	ApA	0.4	0	0.0	0.7	18	0.0
	ApApA	0.4	1899	0.3	0.7	2,280	0.5
	ApApApA	0.3	6080	1.0	0.9	6,380	1.3
	Poly A	0.9	4620	9.3	0.9	3,880	7.4

* Concentrations are expressed in terms of adenosine equivalents. To obtain the concentration as oligonucleotide, divide by the chain length.

† Total number of counts per minute adsorbed onto charcoal.

‡ Micromoles of phosphate incorporated into charcoal-adsorbable compounds per hour per mg. of protein. In the experiment with the *E. coli* enzyme, each tube contained 786,000 c.p.m. as P_i^{32} and 25 γ of protein except for the poly A incubation mixture, which contained 2 γ of protein; incubation time, 1 hour. In the experiment with the *A. vinelandii* enzyme, each tube contained 556,000 c.p.m. as P_i^{32} and 14 γ of protein except for the poly A incubation mixture, which contained 1.5 γ of protein; incubation time, 2 hours. The other components of the reaction mixtures and the procedures are described under the section on methods.

mixtures failed to reveal any significant breakdown of ApApApUp. The remaining data in Tables I and II show that, although the C-3′ phospho monoester moiety inhibits the phosphorolysis of an oligonucleotide, the C-5′ phospho monoester group is not a structural requirement for enzymic action. Thus ApApA and ApApApA (Table I) as well as ApApU and ApApApU (Table II) were phosphorolyzed at rates that do not differ greatly from those found with pApApA and pApApApA. It was also found that the three compounds in Group V, which contain the terminal cyclic-p, were completely resistant to enzymic attack.

Only qualitative conclusions may be drawn from the relative rates of phosphorolysis given in Tables I and II. Insufficient amounts of material made it impossible to conduct the experiments at saturating substrate concentrations and only the following limited conclusions concerning rates can be drawn: (1) with equivalent concentrations of active oligonucleotide, the tetranucleoside derivative is phosphorolyzed more rapidly than is the

TABLE II

Phosphorolysis of Compounds in Group III and IV

Group No.	Substrate	*E. coli* experiment			*A. vinelandii* experiment		
		Substrate concentration*	Total radioactivity†	Rate of phosphorolysis‡	Substrate concentration*	Total radioactivity†	Rate of phosphorolysis‡
		μmole per ml.	*c.p.m.*		*μmole per ml.*	*c.p.m.*	
III	ApUp	0.4	0	0.0	0.7	399	0.0
	ApApUp	0.3	18	0.0	0.8	340	0.0
	ApApApUp	0.3	110	0.0	0.8	1682	0.1
IV	ApU	0.3	0	0.0	0.6	115	0.0
	ApApU	0.3	644	0.2	0.6	4640	0.4
	ApApApU	0.3	2600	0.8	0.3	3145	0.3
	Poly A	0.9	1781	10.4	0.9	3365	5.6

* Concentrations are expressed in terms of nucleoside equivalents. To obtain the concentration, as oligonucleotide, divide by the chain length.

† Total number of counts per minute adsorbed onto charcoal.

‡ Micromoles of phosphate incorporated into charcoal-adsorbable compounds per hour per mg. of protein. In the experiment with the *E. coli* enzyme, each tube contained 544,000 c.p.m. as P_i^{32} and 20 γ of protein except for the poly A incubation mixture, which contained 1 γ of protein; incubation time, 1 hour. In the experiment with the *A. vinelandii* enzyme, each tube contained 628,000 c.p.m. as P_i^{32} and 30 γ of protein except for the poly A incubation mixture, which contained 1.5 γ of protein; incubation time, 2 hours. The other components of the reaction mixtures and the procedures are described under the section on methods.

trinucleoside derivative, (2) the rates of phosphorolysis of corresponding members of the several groups are of the same order of magnitude, as for example pApApA, ApApA, and ApApU. Data on the phosphorolysis of poly A are included as a standard for comparison of rates and were obtained at a concentration of polymer which afforded a maximal reaction rate. Preliminary experiments carried out with pApApA indicated that a concentration of 2.3 μmoles per ml. as adenosine units (0.8 μmole per ml. as trinucleotide) was sufficient to saturate the *E. coli* enzyme. When both were tested at saturating concentrations, the rate of phosphorolysis of pApApA exceeded that found for poly A by a factor of approximately

2-fold. The phosphorolysis of oligonucleotide attained a maximal rate at 10^{-2} M inorganic P and required Mg^{++} ion.

The production of nucleoside diphosphates as one of the products of the phosphorolysis of oligonucleotides was demonstrated by the spectrophotometric assay as well as by the chromatographic evidence discussed below.

TABLE III

Production of Nucleoside Diphosphates from Oligonucleotides

Experiment No.	Substrate	Substrate concentration*	P_i^{32} incorporated into charcoal adsorbable	Nucleoside diphosphate produced
		μmoles per ml.	*μmole*	*μmole*
1	pApA	1.8	0.00	0.00
	pApApA	1.6	0.13	0.14
2	pApApApA	1.4	0.14	0.19
	ApApA	1.3	0.03	0.03
	ApApApA	0.6	0.04	0.04
3	ApU	0.5	0.00	0.00
	ApApU	0.6	0.02	0.02
	ApApApU	0.5	0.04	0.04

* Concentrations are expressed in terms of nucleoside units. To obtain the concentration, as oligonucleotide, divide by the chain length. The incubation mixtures were as described in the section on methods, except that the scale was doubled (final volume, 0.25 ml.). The *E. coli* enzyme was added in the following amounts: 20 γ in Experiments 1 and 2, and 30 γ in Experiment 3. After incubation for 2 hours at 37°, 0.1 ml. was treated as described for the standard assay to determine the micromoles of P_i^{32} incorporated into charcoal-adsorbable compounds. Another 0.1 ml. aliquot was diluted to 0.5 ml. (Experiment 1) or 0.4 ml. (Experiments 2 and 3) with H_2O, heated for 2 minutes at 100°, centrifuged to remove the protein, and 0.30 ml. aliquots of the supernatant fluid were used to determine ADP or UDP. The components of the assay system (1.00 ml. in a cuvette with a 1.0 cm. light path) were 5 μmoles of $MgCl_2$, 0.4 μmole of phosphoenolpyruvate, 0.1 μmole of DPNH, 0.005 ml. of myokinase, and 0.005 ml. of lactic dehydrogenase containing pyruvate kinase.

The data in Table III show that the amount of inorganic P^{32} incorporated into charcoal-adsorbable compounds was equal to the nucleoside diphosphate formed.

It was found that dinucleotides and dinucleoside monophosphates did not undergo phosphorolysis (Tables I, II, and III). This was true regardless of the nature of the end group on the compound containing 2 nucleoside units. A similar conclusion was reached when the phosphorolysis of pApApA and pApApApA was studied as a function of time and the reactions were allowed to go to completion. Fig. 2 shows that poly A was

phosphorolyzed approximately 100 per cent; that is, the inorganic P^{32} incorporated into charcoal-adsorbable nucleotides when the reaction stopped was equivalent to the micromoles of polymer (expressed as adenine residues) originally added. With the trinucleotide, however, inorganic P^{32} uptake ceased when 1 μmole of phosphate had been consumed per 3 μmoles of adenine residues, or, to express this in another way, when 1 phosphate molecule had been incorporated per molecule of trinucleotide (Fig. 2).

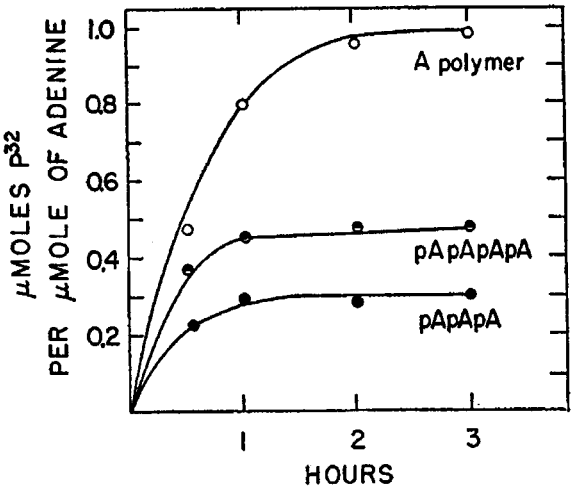


FIG. 2. Phosphorolysis of A polymer, pApApA, and pApApApA. Reaction mixtures were as described in the section on methods but the scale was doubled. The concentrations of poly A, pApApA, and pApApApA were 0.9, 2.5, and 1.1 μmoles of adenine units per ml., respectively. Each tube contained 13×10^5 c.p.m. as P_i^{32}. *E. coli* enzyme was added as follows: 2 γ for poly A, 20 γ for pApApA, and 10 γ for pApApApA. Aliquots (0.05 ml.) of the reaction mixtures were removed at the indicated times. The results are expressed as the ratio of the total number of micromoles of P_i^{32} incorporated into charcoal-adsorbable compounds to the number of micromoles of substrate (as adenine units) present in the incubation.

This suggested that the reaction proceeded according to the following equation

$$\text{pApApA} + P_i \rightarrow \text{pApA} + \text{ADP} \qquad (2)$$

and came to a halt when the limit dinucleotide was formed. Similarly, with the tetranucleotide, the incorporation of inorganic P^{32} ceased when 2 μmoles of phosphate were taken up per micromole of tetranucleotide (Fig. 2), again indicating that the dinucleotide is an end product of phosphorolysis. It should be mentioned that the accumulation of such a limit dinucleotide during the phosphorolysis of poly A would have been too small to detect in this experiment.

A dinucleotide tentatively identified as cyclic dianhydrodiadenylic acid was also tested as a substrate for *E. coli* polynucleotide phosphorylase.

Neither the chemically prepared material (22) nor that obtained from mammalian tissues (23) was phosphorolyzed by the enzyme.[5]

Identification of Reaction Products—Chromatographic investigation of the reaction products confirmed the identification of the nucleoside diphosphates and also showed that a dinucleotide or dinucleoside monophosphate, depending on the starting substance, accumulates during the phosphorolysis of trinucleoside or tetranucleoside derivatives. Reaction mixtures were treated as described under the section on methods and nucleotide material was eluted from the washed charcoal by successive treatments with ethanolic ammonia. The eluates were chromatographed in appropriate solvents, along with suitable markers, and the ultraviolet-absorbing spots were then eluted and treated in various ways to confirm their identity. In one typical experiment, the substrate was pApApApA and the charcoal eluate was chromatographed in solvent System 2. Fig. 3, *a* is a photograph of that chromatogram. The markers are on the left and the reaction mixture on the right. The most rapidly moving area (*c*) contained the dinucleotide pApA and will be discussed in detail. The next area (*b*) corresponds in R_F to ADP and the slowest moving area (*a*) to ATP. ATP arises from ADP owing to myokinase present in the *E. coli* enzyme preparation, and the AMP that is also formed is mixed with pApA in the fastest moving area. The ADP area (*b*) was eluted and its specific radioactivity determined. The data in Table IV give the specific activity of the ADP as well as that of the ADP and UDP isolated by similar techniques from the phosphorolysis of the other oligonucleotides. In each case, the specific radioactivity of the nucleoside diphosphate was equal to that of the inorganic P^{32} present in the incubation mixture. Included in Table IV are the R_F values of authentic samples of the nucleoside diphosphates and those found for the isolated materials. As indicated in Table IV, this procedure did not afford quantitative recovery of the products.

In the experiment with pApApApA (Fig. 3, *a*) the material with the highest R_F in Solvent 2 (Area *c*) was eluted and portions of it were subjected to analysis by Systems 1 and 3. A photograph which demonstrates the paper electrophoresis of this material is shown in Fig. 3, *b*. The known markers are indicated; the eluate from Area *c* of Fig. 3, *a* had the mobility of pApA. In System 1 the major portion of the material also behaved as pApA (Table V). Similar techniques were used to identify pApA as a product of the phosphorolysis of pApApA, and ApA as a product of the phosphorolysis of ApApA, ApApApA, ApApU, and ApApApU (Table V). The solvent system used for the initial separation of the dinucleotide or

[5] We are indebted to Dr. Markham for a sample of the synthetic material and to Dr. Rall for a sample of the isolated compound.

dinucleoside monophosphate from the nucleoside diphosphate is shown in Table V; the material was eluted from the paper and then subjected to the other treatments indicated. The dinucleotides and dinucleoside mono-

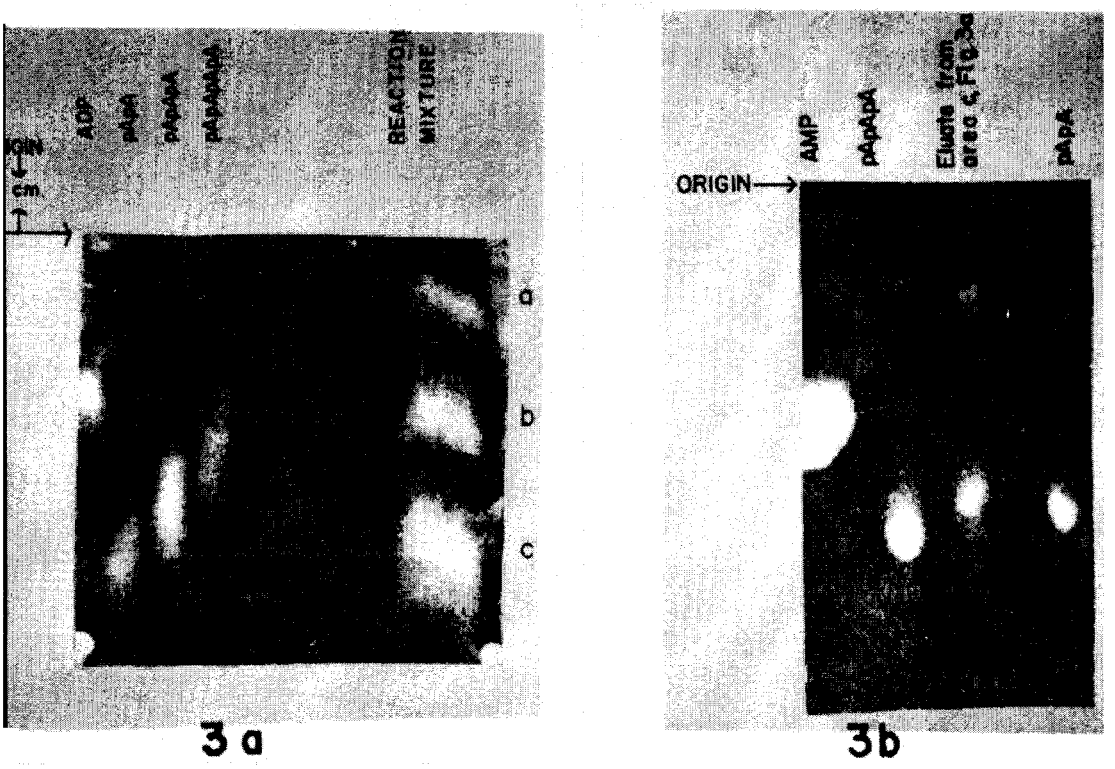


FIG. 3. (*a*), ultraviolet photograph of paper chromatogram showing the products of phosphorolysis of pApApApA. The reaction mixture (0.15 ml. volume) contained 5 μmoles of Tris buffer, pH 8, 0.5 μmole of $MgCl_2$, 3.2 μmoles of P_i^{32} containing 596,000 c.p.m., 20 γ of *E. coli* enzyme, and 0.3 μmole of pApApApA (expressed as adenosine units). Incubation time, 1.5 hours at 37°. The entire mixture was treated as described in the section on methods and the washed charcoal was eluted three times with 0.8 ml. of ethanolic ammonia. The eluate was concentrated, applied to paper, and chromatographed for 20 hours in System 2. The known compounds are on the left. (*b*), ultraviolet photograph of paper electrophoresis strip demonstrating pApA as a product of the phosphorolysis of pApApApA. Area *c* of the chromatogram shown in (*a*) was eluted with water, concentrated, and subjected to paper electrophoresis (System 3) for 2 hours.

phosphates isolated were devoid of any radioactivity. The pApA isolated from the phosphorolysis of pApApA was, in addition, treated with semen monoesterase as described for the preparation of the compounds of Group II. The product ApA was identified by chromatography in Solvent 1, where it had an $R_{5'\text{-AMP}}$ (R_F relative to the R_F of 5'-AMP) equal to 2.23. The $R_{5'\text{-AMP}}$ of known ApA was 2.24, and that for pApA was 0.42.

TABLE IV

Identification of ADP and UDP as Products of Oligonucleotide Phosphorolysis

Substrate		NDP produced	R_F of NDP produced*	R_F of authentic NDP*	NDP eluted	NDP		P_i^{32}†
	Concentration‡							
					μmole	c.p.m.	c.p.m. per μmole	
pApApA.....	0.3	ADP	0.42	0.37	0.024	3,700	150,000	180,000
pApApApA.....	0.3	"	0.36	0.36	0.014	2,900	210,000	190,000
ApApA.....	0.6	"	0.48	0.43	0.041	5,400	130,000	140,000
ApApApA.....	0.5	"	0.30	0.29	0.064	9,700	150,000	150,000
ApApU.....	0.7	UDP	0.17	0.19	0.110	18,400	170,000	160,000
ApApApU.....	0.6	"	0.22	0.22	0.108	16,800	160,000	160,000
		ADP	0.45	0.46	0.045	6,500	140,000	160,000

* R_F values in solvent System 2.

† Specific radioactivity, in counts per minute per micromole, of inorganic P^{32} present in the incubation mixture.

‡ Substrate concentrations are expressed as nucleoside units present in a 0.15 ml. incubation mixture. These experiments were carried out in a manner similar to that described for the experiment of Fig. 3. The *E. coli* enzyme was used. The data for pApApApA here were obtained from that experiment. The nucleoside diphosphate area on the chromatogram was quantitatively eluted, its ultraviolet absorption determined to give the micromoles of ADP produced, and the radioactivity of the eluate was determined on a suitable aliquot. Similar manipulations gave the data for the other compounds.

TABLE V

Identification of Dinucleotides and Dinucleoside Monophosphates as Products of Oligonucleotide Phosphorolysis

Substrate	Limit dinucleoside derivative	System 2*		System 1		System 3†	
		R_F authentic compound	R_F found	$R_{5'\text{-AMP}}$ authentic compound‡	$R_{5'\text{-AMP}}$ found‡	Mobility authentic compound	Mobility found
pApApA	pApA	0.48	0.48	0.40	0.45	9.1	8.9
pApApApA	pApA	0.49	0.47	0.44	0.43	10.9	10.9
ApApA	ApA	0.73	0.73	1.9	1.9	4.7	4.6
ApApApA	ApA	0.67	0.62	2.0	2.0		
ApApU	ApA	0.74	0.70	2.0	2.2	4.0	4.4
ApApApU	ApA	0.74	0.74	2.0	2.2		

* System used to separate dinucleoside derivative from nucleoside diphosphate and nucleoside triphosphate.

† Mobility is calculated as cm. per 2 hours per 1000 volts applied.

‡ $R_{5'\text{-AMP}}$ gives the R_F relative to the R_F of 5'-AMP. The dinucleoside derivative end products were obtained from the same experiments described for Table IV, or from similar experiments. The experiment for pApApApA is described in detail in the text and in Fig. 3. The charcoal eluates were chromatographed in System 2 to separate the products, the pApA and ApA areas were eluted and concentrated, and samples were subjected to chromatography in System 1 and paper electrophoresis.

In one experiment, all the products of phosphorolysis of pApApA were eluted quantitatively from a paper chromatogram run in System 2, and the yield of each substance was determined. It was assumed, for the purpose of calculation, that the pApA was contaminated by an amount of AMP equivalent to the ATP found, since both would have been formed in equal quantities from ADP by the action of myokinase. It was then possible to determine that the ratio of adenylic acid units recovered as pApA to those recovered as the sum of ATP, ADP, and AMP was 2.2. The theoretical value given by Equation 2 is 2.0.

DISCUSSION

The data presented here describe the specificity of polynucleotide phosphorylase with respect to the nature of the end groups on a polynucleotide substrate. It is clear that oligonucleotides which contain a monoesterified phosphate at C-3′ of the terminal nucleoside residue (ApApUp and ApApApUp) are resistant to phosphorolysis, and the same is true for the three oligonucleotides bearing a terminal 2′,3′-cyclic phosphate. By contrast, phosphorolysis occurs readily with comparable compounds which possess phosphate monoesterified at C-5′ (pApApA and pApApApA) or contain no phospho monoester groups at all (for example, ApApA, ApApU).

The resistance of relatively degraded commercial yeast RNA and RNA "core" to phosphorolysis (3) is consistent with the present results, for these preparations are known to contain C-3′ end groups (20, 24). Other factors such as molecular size must be considered, however, when comparing the rates of phosphorolysis of different RNA and biosynthetic polymer preparations. Ochoa has demonstrated that the state of aggregation of the polymer chains influences the rate of phosphorolysis (9). In the present work, comparisons have been made between oligonucleotides of the same chain length, so that the influence of end group structure could be studied without the complication of gross differences in molecular weight.

A study of the products of phosphorolysis of oligonucleotides yields additional information concerning the mechanism of the polynucleotide phosphorylase reaction. The cleavage of the trinucleoside diphosphate, ApApA, can be used as an example. Phosphorolysis can occur in one of the two ways illustrated in Equations 3 and 4:

$$\text{ApApA} + \text{P}_\text{i} \rightarrow \text{ApA} + \text{ADP} \quad (3)$$

$$\text{ApApA} + \text{P}_\text{i} \rightarrow \text{adenosine} + \text{ppApA} \quad (4)$$

Equation 3 involves cleavage at a nucleoside unit linked to the chain by its C-5′-hydroxyl, and the products are the pyrophosphorylated mononucleotide (ADP) and ApA. In Equation 4 phosphorolysis occurs at the

nucleoside linked by its C-3′-hydroxyl to the rest of the polynucleotide, and the products are adenosine and the pyrophosphorylated oligonucleotide, ppApA. In the experiments described above, the products of the phosphorolysis of ApApA were ApA and ADP, indicating that the reaction proceeded according to Equation 3. Similarly, the phosphorolysis of ApApApU produced UDP and ADP, not adenosine and ADP, and the cleavage of the other oligonucleotides also proceeded according to Equation 3. This mechanism represents the reverse of a polymerization mechanism in which the 5′-nucleoside diphosphate units are added to the C-3′-hydroxyls of the acceptor nucleotides with the displacement of inorganic P from the mononucleotide. This phosphorolysis, according to Equation 3, is equivalent to the reverse of the polymerization Mechanism B discussed by Kornberg (25). Polymerization Mechanism A (25) represents the reverse of Equation 4, and does not appear to be applicable to this enzyme.

Equation 1, as written here and by others (2, 3, 6), implies that polynucleotide phosphorylase catalyzes the formation of polynucleotide chains from mononucleotides alone. Assuming this mechanism, it might be expected that in the reverse direction, namely phosphorolysis, cleavage would result in the complete breakdown of a polynucleotide to mononucleotide units. The data presented above demonstrate that this is not the case. The phosphorolysis of oligonucleotides containing 3 or 4 nucleoside residues results in the accumulation of the compounds with 2 nucleoside units. In confirmation, Tables I and II show that pApA, ApA, and ApU are not phosphorolyzed at significant rates.

One possible explanation for these results may be that the enzyme demonstrates an exacting specificity when the substrate presented to it has only 2 nucleoside units. The condensation of 2 ADP molecules according to Equation 1 would be expected to form ppApA, a dinucleotide with a pyrophosphate end group. This compound has not yet been prepared, and it might undergo phosphorolysis.

An alternative conclusion could be that polynucleotide phosphorylase, like starch phosphorylase (26), catalyzes only a limited phosphorolysis of the chain, and the "limit oligonucleotide" happens to be a dinucleotide. Recent experiments (27, 28) have suggested that purified preparations of *A. vinelandii* polynucleotide phosphorylase catalyze the condensation of 2 mononucleotide units to a dinucleotide very slowly, if at all. Thus, a lag in the polymerization reaction can be overcome by the addition of a preformed polynucleotide chain to the reaction mixture. In analogy with the enzymic synthesis of polysaccharides, the new polymer has been shown to be built onto this primer. It is consistent with the experiments reported here that the primer may be as small as a dinucleotide (28).

The author wishes to thank Dr. Leon A. Heppel for suggesting this problem and for many stimulating discussions during the course of the work.

SUMMARY

The phosphorolysis of oligonucleotides by preparations of polynucleotide phosphorylase from *Azotobacter vinelandii* and *Escherichia coli* has been studied. Tri- and tetranucleotides with a phospho monoester group at the terminal C-5′ were readily phosphorolyzed; however, if the phospho monoester group was at the terminal C-3′, the compounds were resistant to enzymic attack. Trinucleoside diphosphates and tetranucleoside triphosphates were also phosphorolyzed by these enzymes, indicating that the C-5′ phospho monoester moiety is not required in order that an oligonucleotide be a substrate. Dinucleotides and dinucleoside monophosphates were not phosphorolyzed at significant rates. The products of the phosphorolysis of the compounds with 3 and 4 nucleoside residues were identified as the nucleoside diphosphates (ADP or UDP) and the resistant dinucleotide or dinucleoside monophosphate.

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